IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: GUY, John. Confirmation No: 9515

Application No.: 10/687,677 Examiner: SHEN W. C. W.

Date Filed: October 17, 2003 Group: 1632

For: REDUCING CELLULAR DYSFUNCTION CAUSED BY MITOCHONDRIAL GENE MUTATIONS

DECLARATION UNDER 37 C.F.R. §1.131
OF JOHN GUY

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, John Guy, hereby declare:

1. I am over the age of 21, and I am competent to make this Declaration based upon my personal knowledge. I understand that this Declaration will be used in the United States Patent and Trademark Office ("Patent Office") in connection with the above-identified patent application. I understand that this Declaration is being submitted in order to show that the invention claimed in the above-identified application was conceived in the United States before the following publications: Guy, Gene therapy for nuclear complementation of the G11778A LHON mitochondrial DNA mutation, *Neurology*, (April 24, 2001) Vol. 56, No. 8 Supplement 3, pp. A14. print. Meeting Info.: 53rd Annual Meeting of the American Academy of Neurology. Philadelphia, PA, USA. May 05-11,2001. American Academy of Neurology. CODEN: NEURAI. ISSN: 0028-3878); Guy et al., *Ann Neurol* 52(5): 534-42,2002, published online October 11 2002; Guy et al., Gene therapy with the ND4 subunit gene recoded in the universal genetic code reverses a mitochondrial deficiency causing Leber Hereditary Optic Neuropathy

(LHON), Neurology, (April 9, 2002) Vol. 58, No. 7 Supplement 3, pp. A508. print. Meeting Info.: 54th Annual Meeting of the American Academy of Neurology. Denver, Colorado, USA. April 13-20, 2002. CODEN: NEURAI. ISSN: 0028-3878.

2. I am the inventor who conceived and diligently pursued the above-referenced invention in the United States prior to the earliest publication dates of: Guy, Gene therapy for nuclear complementation of the G11778A LHON mitochondrial DNA mutation, *Neurology*, (April 24, 2001) Vol. 56, No. 8 Supplement 3, pp. A14. print. Meeting Info.: 53rd Annual Meeting of the American Academy of Neurology. Philadelphia, PA, USA. May 05-11,2001. American Academy of Neurology. CODEN: NEURAI. ISSN: 0028-3878); Guy et al., *Ann Neuro*l 52(5): 534-42,2002, published online October 11 2002; Guy et al., Gene therapy with the ND4 subunit gene recoded in the universal genetic code reverses a mitochondrial deficiency causing Leber Hereditary Optic Neuropathy (LHON), Neurology, (April 9, 2002) Vol. 58, No. 7 Supplement 3, pp. A508. print. Meeting Info.: 54th Annual Meeting of the American Academy of Neurology. Denver, Colorado, USA. April 13-20, 2002. CODEN: NEURAI. ISSN: 0028-3878.

3. Attached as Exhibit A are copies of my laboratory notebooks date March 13, 2001 showing: (1)The completion of packaging of the nuclear encoded ND4 subunit with appended FLAG epitope into the viral vector after more than 1 year of work in constructing this approximately 1500 bases from overlapping 80 mer oligonucleotides. (2).Demonstration of ND4Flag expression by western immunobloting of homogenates from infected cells. (3) .Immunofluorescent microscopy of mitochondrial localization of ND4FLAG fusion protein using MitoTracker Red in transfected cell culture.

4. These documents demonstrate that I originally conceived and worked diligently since Feb 2000 to generate the nuclear encoded ND4 data of this patent application.

5. Attached as Exhibit A is a copy of the experiments conducted before the publication date of Guy, Gene therapy for nuclear complementation of the G11778A LHON mitochondrial DNA mutation, *Neurology*, (April 24, 2001) Vol. 56, No. 8 Supplement 3, pp. A14. print. Meeting Info.: 53rd Annual Meeting of the American Academy of Neurology. Philadelphia, PA, USA. May 05-11,2001. American Academy of Neurology. CODEN: NEURAI. ISSN: 0028-3878); Guy et al., *Ann Neuro*l 52(5): 534-42,2002, published online October 11 2002; Guy et al., Gene therapy with the ND4 subunit gene recoded in the universal genetic code reverses a mitochondrial deficiency causing Leber Hereditary Optic Neuropathy (LHON), Neurology, (April 9, 2002) Vol. 58, No. 7 Supplement 3, pp. A508. print. Meeting Info.: 54th Annual Meeting of the American Academy of Neurology. Denver, Colorado, USA. April 13-20, 2002. CODEN: NEURAI. ISSN: 0028-3878.

6. As indicated, the purpose of the experiments were (1) Construction and packaging of the nuclear encoded ND4 subunit with appended FLAG epitope into the viral vector. (2).Demonstration of ND4Flag expression by western immunobloting of homogenates from infected cells. (3) .Immunofluorescent microscopy of mitochondrial localization of ND4FLAG fusion protein using MitoTracker Red in transfected cell culture. (4) Rescue of the defective respiration of cybrid cells containing mutated ND4 mitochondrial DNA. These experiments describe the claimed invention with sufficient specificity to enable a person having ordinary skill in the art to practice the claimed invention.

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7. Attached as Exhibit B is a copy of the filing receipt of the Provisional patent application which indicates that I was diligent in applying for a patent application once I had reduced the invention to practice. The provisional application was filed on October 18, 2002 by the University of Florida and was awarded serial number 60/419,435.

8. In light of the above, I submit that reasonable diligence was exercised from the initial conception of the invention with continued efforts to diligently practice the invention as evidenced by the body of work, attached as Exhibit A. To the best of my knowledge, I initially conceived of the invention around 1997. I diligently proceeded towards reducing the invention to practice during the period from March 2000 through the filing of US Provisional Patent Applications: 60/271,073, filed February 23, 2001; US Provisional Patent Application 60/275,288, filed March 12, 2001; and, U.S. Patent Application No. 10/164,363 on June 6, 2002.

9. I further state that all statements made herein are true and that all statements made on information and experimental results under their experimental conditions are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 10-05-07

John Guy, M.D.

EXHIBIT "A"

① VIRAL constitut made + titd

VIRUS (Bay ± S)
ALD4-8 250 ml 2.0 × 10^13
(RND4) RND4-F 250 ml 7.0 × 10^13

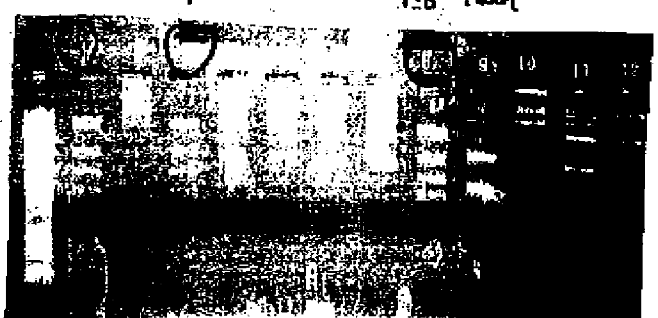
Fugene transfect ND4-UF, today in OVEN
5 FBS 6h [illegible] media.

3/13/01

minipp A_6-11

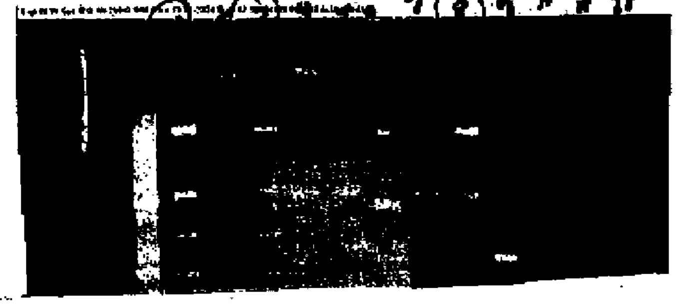
A_6-11 [illegible] 3/14/01

E 1,3 + 8 have Ab [illegible]



A_6-11 A_6 EcoR1
5'→3' 842, 1150, 1758, 4327
3'→5' [illegible]

3/14 A_6-11



4 bolo
6960, 7100, 7600, 7400 all 5'→3'

large plasmid prep #①

3/15/01 ② Western shows expression

Western blot of ND4-11 Fugene transfect
combined all 3 wells vs 3 wells control
saw ⊕ band at 1/10 dilution of cell lysate.

3/26/01 ③ Immunocytochem shows expression

	ND4-11	ND4-12	ND4-GFP	Fugene
1°	flag mouse SFP rabbit	flag mouse SFP rabbit	flag mouse SFP rabbit	O/N
2°	Cy2-mouse	Cy5 mouse Cy2 rabbit	Cy2 rabbit	

ok NT [illegible] none F/4 Cy2 rabbit

Fugene trans 3:1 ND4 × 6 well

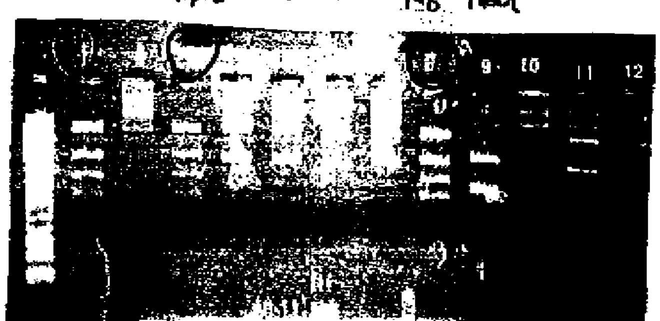
SEP-26-2007 WED 04:35 PM 3928554 FAX NO. 3523927839 P. 02/06

Virus (Bay #5)
ALO4-8 250 ul 2.0 x 10^13
(Rodge) RND4-F 250 ul 3.0 x 10^13

Fugene transfect ND4-LIF, today in OVEN
5 PBS + 6h Ag cell media.

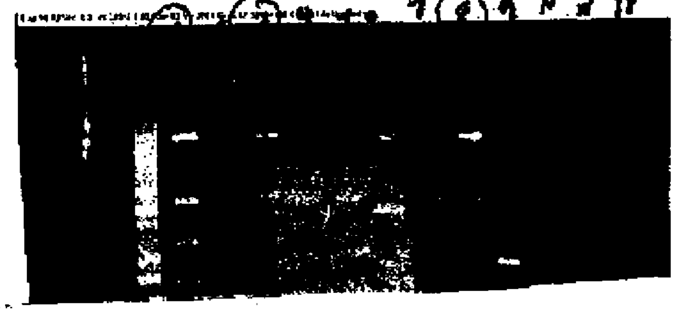
3/13/01
minprep A6-11

A6-11 + Bsr dilut 3/14/01
113 + 8 hou A6 insert



A6-11 5'→3' A9 EcoR1 892, 1150, 1758, 4327
3'→5'

3/14 A6-11



4 bolo
6960, 7100, 7160, 7400 all 5'→3'
large plasmid prep of #1

3/15/01
Western blot of ND4-11 Fugene transfect
control NI 3 wells vs 3 wells control
SDS + bars at 1/10 dilution of cell lysate.

3/26/01

	ND4-11	ND4-12	ND4-GFP	Fugene
P	flag mouse SEP rabbit	flag mouse SEP rabbit	flag mouse SEP rabbit	O/N
2°	CY2-mouse	CY5 mouse CY2 rabbit	CY2 rabbit	

Ab NT 488N opt, none F/A CY2 rabbit

Fugene trans 3:1 ND4 x 6 well

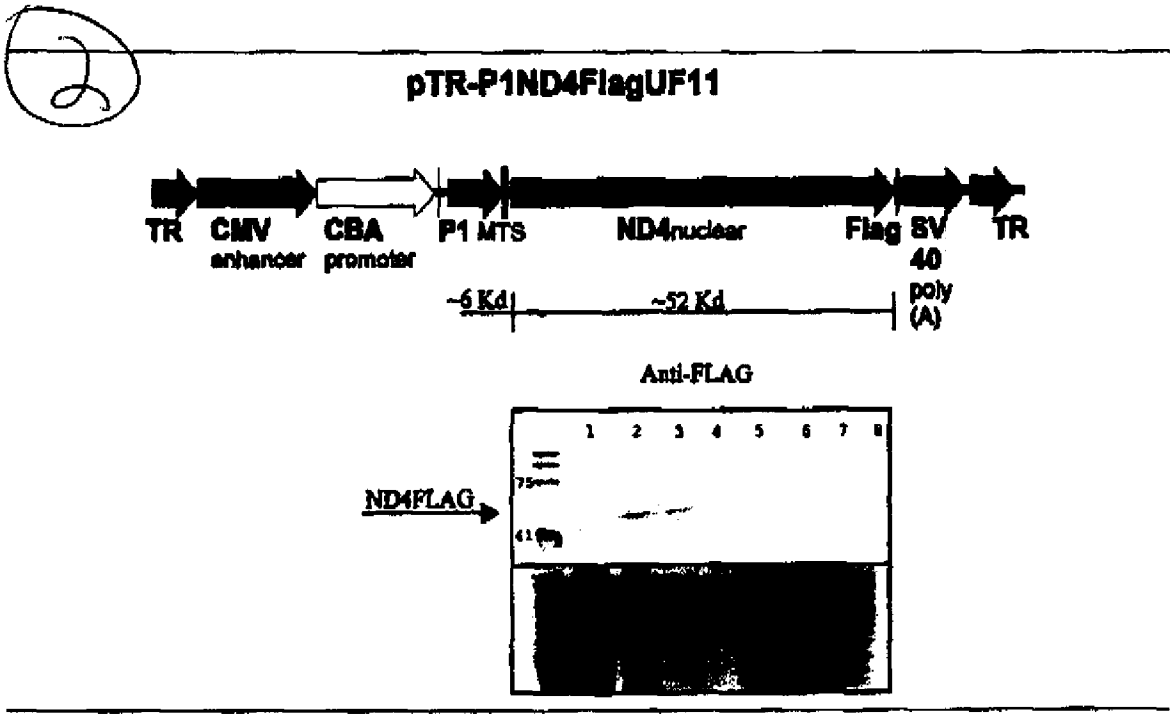


Fig 1. Illustration and immunoblotting of the P1ND4FLAG construct in UF-11. Diagram showing the nuclear-encoded ND4 in AAV vector UF-11 (top). Cellular infection with this construct should result in the synthesis of a 52kD polypeptide, the molecular weight of the ND4Flag. (bottom) Western blot of ND4Flag-transfected G11778A cybrids (lanes 1–4) shows a 52kD band consistent with expression of the ND4Flag fusion polypeptide (lanes 2, 3), whereas the control (untransfected cells; lanes 5–8) shows no staining with the anti-FLAG antibody. Stained gel shows corresponding protein loading with successive 1 log unit dilutions (bottom half). Overloading of lane 1 by cellular protein is readily apparent by the absence of any discrete pattern of protein bands in the stained gel. This is in contrast with lane 2 in which discrete bands are best seen and the intensity of anti-Flag immunostaining was optimized. CMV = cytomegalovirus; TR = terminal repeat; CBA = chicken B actin.

cytoplasmic GFP in the same cell. Cells mock- transfected with AAV vector UF-11 driving GFP expression in the place of the P1ND4Flag gene exhibited diffuse cytoplasmic staining of GFP only (see Fig 2H). Last, when ND4 with the Aldh MTS was linked to GFP, rather than to FLAG, the ND4GFP fusion did have a punctate staining pattern mimicking import into mitochondria (see Fig 2I), but relatively poor colocalization of GFP with MitoTracker Red (see Fig 2I) suggested this fusion protein was not imported.

Allotopic ND4 Improves Cybrid Cell Survival

Although P1ND4Flag was expressed and imported into mitochondria, would allotopic complementation with this protein improve the defective oxidative phosphorylation of LHON? To answer this question, homoplasmic cybrid cells harboring mutant mtDNA (ie, 100% G11778A derived from a patient with LHON inserted into a neutral nuclear background) were transfected with rAAV containing the P1ND4Flag or mock-transfected with the same AAV plasmid lacking the allotopic insert and expressing GFP (UF-11). Immediately after the transfection, cells were grown in glucose-rich media for 3 days and then placed in glucose-free media containing galactose as the main carbon source for glycolysis. This media forces the cells to rely predominantly on oxidative phosphorylation to produce ATP.[27] Cells harboring complex I mutations have a severe growth defect compared with wild-type cells in such medium.[26] We found that cybrid cell survival after 3 days in the glucose-deficient galactose media was threefold greater for the allotopically transfected P1ND4Flag cybrids than were the cybrids transfected with the mock AAV ($p < 0.001$; Fig 3A). Apparently, in the mutated cybrids this selection enriched for cells that expressed higher levels of P1ND4Flag, suggesting these cells likely had improved oxidative phosphorylation.

Oxidative Phosphorylation Deficiency Rescued by Allotopic ND4

Consistent with the finding that spectrophotometric assays of complex I activity do not discriminate between wild-type cells and G11778A mutant cybrids,[9–11,28,29] transfection with P1ND4Flag did not increase complex I activity (see Fig 3B). These results are in accord with published observations that the impact of the G11778A LHON mutation on complex I–specific activity in cell

3

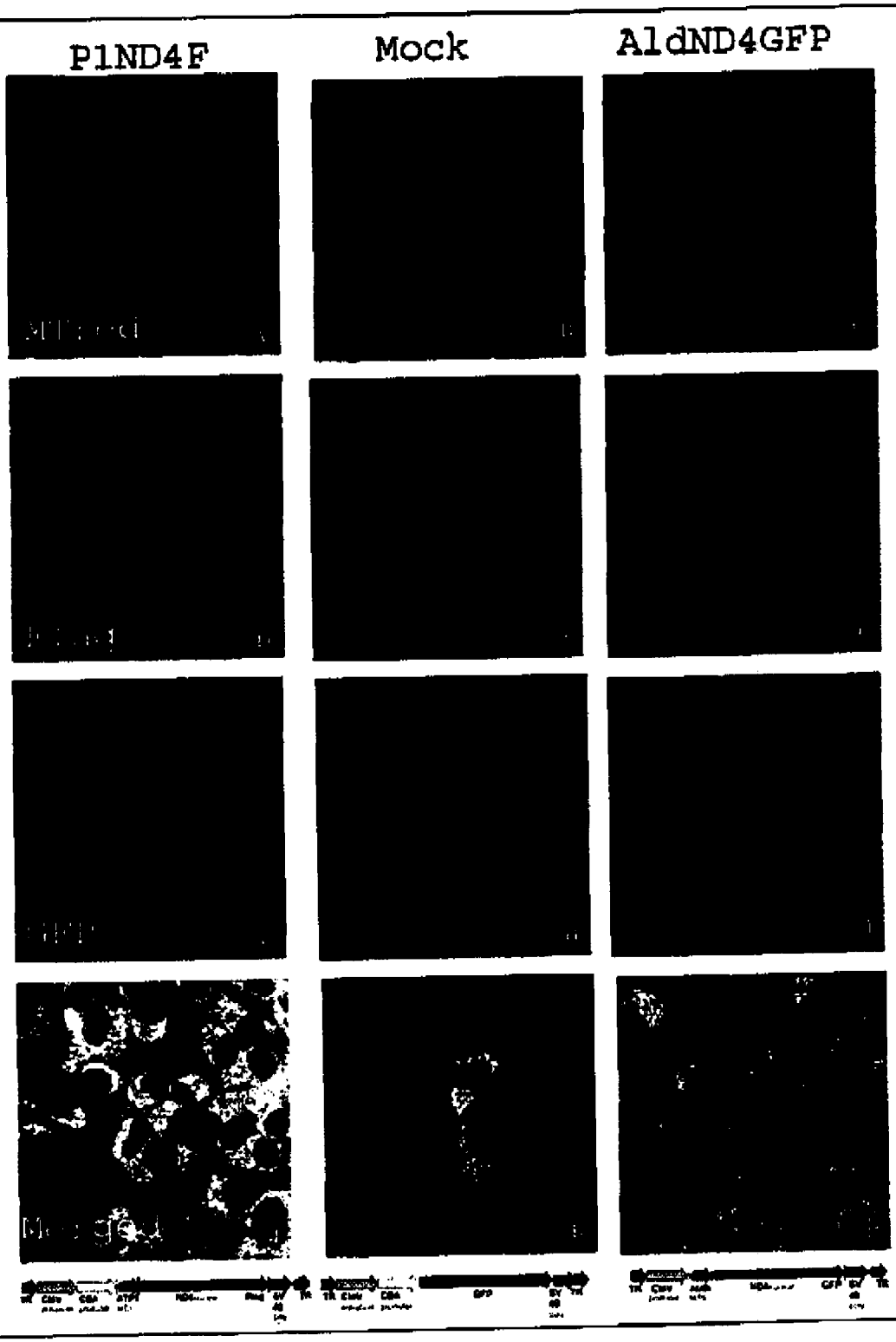


Figure 2

ORIGINAL ARTICLES

Rescue of a Mitochondrial Deficiency Causing Leber Hereditary Optic Neuropathy

John Guy, MD,[1,2] Xiaoping Qi, MD,[1] Francesco Pallotti, MD, PhD,[3] Eric A. Schon, PhD,[3] Giovanni Manfredi, MD,[4] Valerio Carelli, MD, PhD,[5] Andrea Martinuzzi, MD, PhD,[6] William W. Hauswirth, PhD,[1,7] and Alfred S. Lewin, PhD[7]

A G to A transition at nucleotide 11778 in the ND4 subunit gene of complex I was the first point mutation in the mitochondrial genome linked to a human disease. It causes Leber Hereditary Optic Neuropathy, a disorder with oxidative phosphorylation deficiency. To overcome this defect, we made a synthetic ND4 subunit compatible with the "universal" genetic code and imported it into mitochondria by adding a mitochondrial targeting sequence. For detection we added a FLAG tag. This gene was inserted in an adeno-associated viral vector. The ND4FLAG protein was imported into the mitochondria of cybrids harboring the G11778A mutation, where it increased their survival rate threefold, under restrictive conditions that forced the cells to rely predominantly on oxidative phosphorylation to produce ATP. Since assays of complex I activity were normal in G11778A cybrids we focused on changes in ATP synthesis using complex I substrates. The G11778A cybrids showed a 60% reduction in the rate of ATP synthesis. Relative to mock-transfected G11778A cybrids, complemented G11778A cybrids showed a threefold increase in ATP synthesis, to a level indistinguishable from that in cybrids containing normal mitochondrial DNA. Restoration of respiration by allotopic expression opens the door for gene therapy of Leber Hereditary Optic Neuropathy.

Ann Neurol 2002;52:534–542

A G to A transition at nucleotide 11778 in mitochondrial DNA (mtDNA) in the gene specifying the ND4 subunit of complex I results in an arginine to histidine substitution at amino acid 340. It was the first mtDNA point mutation linked to a maternally inherited human disease, Leber hereditary optic neuropathy (LHON), a disorder blinding patients during the second and third decades of life. Since this discovery 14 years ago,[1] more than 30 other pathogenic point mutations in human polypeptide-coding mtDNA genes have been described. Although mtDNA encodes 13 of the proteins needed for oxidative phosphorylation, the remainder are nuclear-encoded proteins that are synthesized on cytoplasmic ribosomes and are imported into the mitochondria, usually directed by an N-terminal mitochondrial targeting presequence.[2] Thus, mutations in either mtDNA or nuclear DNA may impair mitochondrial function, resulting in human disease.[3]

Of all mitochondrial diseases, LHON is the most common.[4] Three mtDNA mutations (G3460A, G11778A, and T14484C) account for 95% of LHON cases, with the G11778A mutation being the most common, accounting for 50% of LHON cases.[4,5] Each LHON mutation affects a different subunit of the nicotinamide adenine dinucleotide:ubiquinone oxidoreductase (complex I) in the oxidative phosphorylation pathway, where electrons first enter the electron transport chain.[6] This large enzyme consists of seven subunits (ND1, 2, 3, 4, 4L, 5, and 6) encoded by mtDNA whereas the remaining 35 subunits are nuclear encoded.[7] Mitochondrial oxidative phosphorylation deficiency due to mutations in complex I subunit genes is believed to play a pivotal role in development of LHON, although the precise pathophysiological events precipitating acute visual failure and cellular injury remain elusive. Each LHON mutation alters mtDNA-encoded intrinsic complex I membrane proteins, but surprisingly the standard spectrophotometric assays of complex I activity in LHON cells containing the G11778A mutation in the ND4 subunit gene are reduced slightly.[8–11] Only the G3460A mutation in the ND1 subunit gene reduces complex I activity markedly.[9,11,12] However, clear evidence of complex I defi-

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Received Apr 11, 2002, and in revised form Jun 20. Accepted for publication Jul 1, 2002.

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EXHIBIT "B"

UNITED STATES PATENT AND TRADEMARK OFFICE

Commissioner for Patents
Washington, DC 20231
www.uspto.gov

APPLICATION NUMBER	FILING DATE	GRP ART UNIT	FIL FEE REC'D	ATTY.DOCKET.NO	DRAWINGS	TOT CLAIMS	IND CLAIMS
60/419,435	10/18/2002		80	5853-324	3		

CONFIRMATION NO. 2175

Stanley A. Kim, Ph.D., Esq.
Akerman, Senterfitt & Eidson, P.A.
222 Lakeview Avenue, Suite 400
P.O. Box 3188
West Palm Beach, FL 33402-3188

FILING RECEIPT

OC000000009177876

Date Mailed: 12/02/2002

Receipt is acknowledged of this provisional Patent Application. It will not be examined for patentability and will become abandoned not later than twelve months after its filing date. Be sure to provide the U.S. APPLICATION NUMBER, FILING DATE, NAME OF APPLICANT, and TITLE OF INVENTION when inquiring about this application. Fees transmitted by check or draft are subject to collection. Please verify the accuracy of the data presented on this receipt. **If an error is noted on this Filing Receipt, please write to the Office of Initial Patent Examination's Filing Receipt Corrections, facsimile number 703-746-9195. Please provide a copy of this Filing Receipt with the changes noted thereon. If you received a "Notice to File Missing Parts" for this application, please submit any corrections to this Filing Receipt with your reply to the Notice. When the USPTO processes the reply to the Notice, the USPTO will generate another Filing Receipt incorporating the requested corrections (if appropriate).**

Applicant(s)

John Guy, Gainesville, FL;

If Required, Foreign Filing License Granted: 11/29/2002

Projected Publication Date: None, application is not eligible for pre-grant publication

Non-Publication Request: No

Early Publication Request: No

**** SMALL ENTITY ****

Title

Reducing cellular dysfunction caused by mitochondrial gene mutations

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Title 37, Code of Federal Regulations, 5.11 & 5.15

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